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Cell death induced by vaccine adjuvants containing surfactants

Ya-Wun Yang [a,*], Ching-An Wu [a], W.J.W. Morrow [b]

[a] School of Pharmacy, College of Medicine, National Taiwan University, Room 1214, 1, Jen-Ai Road, Section 1, Taipei 100, Taiwan
[b] Washington National Primate Research Center and Dept. of Pathobiology, University of Washington, 3000 Western Avenue, Seattle, WA 98121-1023, USA

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Abstract

Many vaccine adjuvants contain surface-active agents, but the immunological roles played by these components have been essentially ignored. The objective of this study was to examine possible apoptotic and necrotic effects of the surface-active agents, Pluronic L121 and Tween 80, which are components of L121-adjuvant (a formulation we synthesized with the aim of representing several commercially produced adjuvants), on EL4 lymphoma cells. Cell viability and cytolytic effects were analyzed using the MTT and LDH release assays, and the distribution of cells in different stages of the cell cycle after treatment with these agents was analyzed by propidium iodide (PI) staining and flow cytometry. L121-adjuvant was shown to induce cell cycle arrest and inhibit cell proliferation. Treatment of EL4 cells with surface-active agents resulted in a concentration-dependent increase in the apoptotic/necrotic cell populations. Fluorescence microscopy using Hoechst 33342 staining demonstrated chromosome condensation and DNA fragmentation in cells treated with surfactants or adjuvant. The apoptotic and necrotic effects of vaccine adjuvant containing surface-active agents were confirmed by Annexin V/propidium iodide staining and flow cytometric analysis. Pretreatment of EL4 cells with zVAD-fmk, a broad range caspase inhibitor, partially prevented apoptosis induced by Pluronic L121, but did not prevent the cell death induced by Tween 80 or L121-adjuvant. These findings suggested that Tween 80 and L121-adjuvant induced apoptosis in EL4 cells via a "non-classical" caspase-independent pathway. Results presented in this study suggest mechanisms of elicitation of $CD8^+$, class I-restricted CTL response by soluble antigens mediated by the vaccine adjuvant containing surface-active agents.

Keywords: Adjuvants; Apoptosis; Cell death; Necrosis; Surfactants

1. Introduction

Vaccine adjuvants were designed to increase the immunological effect of antigens, but the mechanism by which adjuvants enhance the immune response to protein antigens has never been clearly elucidated. For over 75 years since the first report of the use of alum precipitates [1], extensive research has been carried out on the development of vaccine adjuvants, yet alum still remains the only approved adjuvant for clinical human use today. Since the development in the 1930s of Freund's adjuvant, in which tubercle bacilli and paraffin oil are used [2], vaccine adjuvants for animal use have often been formulated as disperse systems consisting of at least two phases, i.e., oil and water, and thus require the presence of surface-active agents to stabilize the dispersion by reducing the interfacial tension between the dispersed phase and the dispersion medium. However, the possible immunological roles of these surface-active agents have been ignored. In addition to their effect on the amplitude and duration of the antibody response, vaccine adjuvants have the ability to generate delayed-type hypersensitivity (DTH) and induce class I-restricted cytotoxic T lymphocytes (CTLs) [3–5]. It was therefore suggested that adjuvants help deliver antigen into the cytoplasm via the endogenous pathway or help antigen-presenting cells (APCs) to take up antigens [4], but these ideas have never been proven.

Recent studies have shown that apoptosis can be induced by treatment of cells with sublytic concentrations of non-ionic detergents, such as Triton X-100 or Nonidet P-40 [6]. Apoptosis is an active form of cell death [7]. In mammalian cells, it is often associated with various morphological and structural alterations, characterized by various biochemical changes, including cell shrinkage, phosphatidylserine (PS) exposure, chromatin condensation, and nuclear DNA fragmentation [8]. Apoptotic cell death is important in triggering and regulating the immune response. Studies have

Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic T-lymphocyte; DCs, dendritic cells; DI, deionized water; L121, Pluronic L121; LDH, lactate dehydrogenase; MHC, major histocompatibility complex; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; PS, phosphatidylserine

* Corresponding author. Tel.: +886-2-23918952; fax: +886-2-23919098.
E-mail address: ywyang@ha.mc.ntu.edu.tw (Y.-W. Yang).

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shown that apoptotic cells can be engulfed by macrophages or phagocytosed by dendritic cells (DCs), the most potent APCs, and that only DCs can cross-present exogenously derived antigens on major histocompatibility complex (MHC) class I molecules to CTLs [9–11]. Sauter et al. [12] have shown that immature DCs located in the periphery can efficiently capture apoptotic and necrotic cells, but only exposure to necrotic cells induces DC maturation. Optimal cross-presentation of antigens to $CD8^+$ T cells was therefore suggested to require two steps, the phagocytosis of apoptotic cells by immature DCs and DC maturation induced by exposure to necrotic cells. These findings raised the possibility that immature DCs process exogenous cell-associated antigens from adjuvant-induced apoptotic cells and cross-present them to CTLs, i.e., cross-priming or cross-presentation, while adjuvant-induced necrosis leads to DC maturation and upregulation of maturation signals. In addition, PS, which appears on the cell membrane during apoptosis of thymocytes, is recognized by macrophages [13,14]. The apoptotic and necrotic responses induced by surfactants in vaccine adjuvants have therefore been suggested to play important roles in the immunological effects elicited by surfactant-containing vaccine adjuvants.

To test this hypothesis, a vaccine adjuvant containing Pluronic L121, Tween 80, squalane, and phosphate-buffered saline (PBS), was examined in the present study for the apoptotic and necrotic potential of the individual components present in the formulation. This adjuvant was termed "L121-adjuvant", which resembles Provax™ adjuvant (IDEC Pharmaceuticals, San Diego), an emulsion adjuvant previously shown to elicit CTL responses [4,5,15,16]. The emulsifying agents used in this adjuvant are the two non-ionic surfactants, Pluronic L121 and Tween 80. Pluronic L121 is a triblock copolymer consisting of the hydrophilic poly(ethylene oxide) (PEO) blocks and the hydrophobic poly(propylene oxide) (PPO) block with an hydrophilic–lipophilic balance (HLB) value of 1. The average molecular weight of Pluronic L121 is about 4400, and its approximate chemical formula is $(PEO)_5$-$(PPO)_{68}$-$(PEO)_5$. This copolymer was used in early studies by Hunter's group and later included in the Syntex adjuvant formulation (SAF) [3,17], which was shown to elicit both protective humoral responses and cell-mediated immunity. The biological activities of these adjuvants were attributed to the adsorptive and adhesive properties of the block copolymer [18]. Tween 80 is a polyoxyethylene sorbitan monooleate ester with a POE content of approximately 20 per molecule and an HLB value of 15. This surfactant has often been included in other vaccine formulations, such as Chiron MF59 adjuvant [19,20].

To determine whether surfactant-containing vaccine adjuvants have an apoptotic effect, EL4 cells were treated with the whole adjuvant or with the individual components and tested for cell viability using MTT reduction and LDH release assays. The cell cycle after treatment with the test agents was analyzed using propidium iodide (PI) staining. Apoptosis and necrosis were analyzed quantitatively by staining with Annexin V-FITC, a compound with a high affinity for PS, and with propidium iodide. The results, showing that the surfactant-containing vaccine adjuvant induced both apoptosis and necrosis, have implications for better manipulation of the immune response using vaccine adjuvants for cross-presentation of exogenously derived soluble antigens to CTLs.

2. Materials and methods

2.1. Cells

The EL4 cells, a mouse thymoma cell line derived from C57BL/6-Ly5.2 mice [21], were obtained from the American Tissue Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% horse serum.

2.2. Materials

Pluronic L121, a triblock amphiphilic copolymer was kindly provided by BASF Corp. (Mount Olive, NJ). Ovalbumin and Tween 80 were purchased from Sigma Chemicals (St. Louis, MO). L121-adjuvant, consisting of 3.75% (w/v) Pluronic L121, 0.6% (w/v) Tween 80, and 15% (w/v) squalane in PBS, was prepared according to the published formula [4]. The mixture was sonicated at 4 °C for 5 min by a Branson Sonifier 450 Model (Branson Ultrasonics Corp., Danbury, CT), then passed six times at 12,000 psi through a M-110EH Microfluidizer® Processor (Microfluidics Corporation, Newton, MA). The emulsion was stored at 4 °C until use. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was obtained from Sigma (St. Louis, MO).

2.3. Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay, which measures the conversion of MTT into the purple-colored MTT formazan by the succinate-tetrazolium reductase system in living cells. The decrease in cellular MTT reduction indicates the extent of cell damage [22]. In brief, triplicate cultures of 1×10^5 EL4 cells in 100 μl of DMEM in 96-well plates were incubated for 24 h at 37 °C with the test agents, then 10 μl of stock MTT solution (5 mg/ml) was added to each well, and the plates incubated at 37 °C for 1 h. One hundred microliters of 10% Triton X-100 was added, then the plates were incubated at 37 °C for another 10 min and the optical density of the wells read at 550 nm on a SPECTRAmax PLUS microplate reader (Molecular Devices Corp., USA). The results were analyzed using the SOFTmax PRO program.

2.4. Assay of necrosis using lactate dehydrogenase (LDH) release

The amount of LDH released from lysed cells is a sensitive measure of cell death [23,24]. In this study, necrotic cell death was measured in 96-well plates using the CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI) [24,25]. Briefly, triplicate cultures of 5×10^3 cells in 100 μl of DMEM were incubated for 24 h at 37 °C with various concentrations of the test agents, then centrifuged at $236 \times g$ for 5 min. Fifty microliters of the supernatant was transferred to a flat-bottom plate, and an equal volume of reconstituted Substrate Mix added. The plates were incubated at room temperature for 30 min, then the reaction was stopped by addition of 50 μl of Stop Solution, and the LDH-mediated conversion of the tetrazolium salt into the red formazan product measured at 490 nm.

2.5. Cell cycle analysis and hypodiploid nuclei detection

Cell viability was measured using 4% Trypan blue. For detection of apoptosis induced by the adjuvant or surfactants, the leakage of fragmented DNA from apoptotic cells was measured by a modified Nicoletti method [26]. Briefly, 1×10^6 EL4 cells in 1 ml of DMEM in 12-well plates were incubated at 37 °C for 24 h with the various test agent. The medium was removed and the cells were fixed in 70% ethanol at −20 °C for at least 30 min, then the ethanol was removed and 200 μl of phosphate buffer containing 0.2 M Na_2HPO_4, 0.1 M citric acid (pH 7.8) were added for 30 min at room temperature. The cells were centrifuged and stained for 30 min at room temperature with 1 ml of Propidium iodide (PI) staining buffer (1% Triton X-100, 100 μg/ml of RNAse A, and 80 μg/ml of PI in PBS) and analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA). Cells containing hypodiploid DNA were considered as apoptotic.

2.6. Determination of early apoptosis and necrosis by flow cytometry

To further determine the extent of early apoptosis and necrosis, cell death was analyzed by staining the cells with Annexin V-FITC (Ann V) and PI (1 μg/ml) using the Annexin V-FITC kit (Bender MedSystems, Vienna, Austria). For staining, 1×10^6 EL4 cells per ml in the 12-well plates were treated with various test agents for 24 h, then washed with cold PBS, centrifuged, and suspended in a final volume of 210 μl binding solution (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 5 μl of Annexin V-FITC and 10 μl of PI stock solution (20 μg/ml), as provided by the manufacturer. The cells were incubated at room temperature for 10 min then 400 μl of Hank's balanced salt solution (HBSS) was added and the cells were analyzed using a FACSCalibur flow cytometer. Excitation was at 488 nm and the emitted green fluorescence of Annexin V (FL1) and red fluorescence of PI (FL2) were collected using a 525 and 575 nm band pass filter, respectively. A total of at least 10,000 cells was analyzed per sample. Light scatter was measured on a linear scale of 1024 channels and fluorescence intensity on a logarithmic scale. The amount of early apoptosis and necrosis was determined, respectively, as the percentage of Ann V^+/PI^- or Ann V^+/PI^+ cells.

2.7. Effect of a caspase inhibition by zVAD-fmk

Cells (1×10^6/ml) were incubated for 2 h at 37 °C with 50 μM zVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) (R&D Systems Inc., Minneapolis, MN), a general caspase inhibitor, then the various test agents were added and incubation continued for 24 h at 37 °C. Approximately 1×10^5 cell were then stained with Annexin V-FITC/PI and subjected to flow cytometric analysis. Apoptotic positive control cells were generated by treatment with 1 μM staurosporine for 24 h and necrotic positive control cells by treatment for 24 h at 37 °C with 0.3% H_2O_2 in DMEM.

2.8. Examination of apoptosis by fluorescence microscopy

To examine microscopically the apoptosis and necrosis induced by surface-active agents, 1×10^6 EL4 cells per ml in the 12-well plates were treated for 24 h at 37 °C with 5 mg/ml of Pluronic L121, 1 mg/ml of Tween 80, or 30 μl/ml of L121-adjuvant, then stained for 15 min at 37 °C with 0.1 μg/ml of Hoechst 33342. The cells were then centrifuged, washed with PBS, and fixed for 15 min in 4% paraformaldehyde. After a further wash with PBS, the cells were placed on glass slides, mounted, and examined under a Leica TCS SP2 confocal spectral microscope, equipped with a UV laser 351 nm/364 nm.

2.9. Statistical analysis

Results are presented as the means±standard errors (S.E.) for three separate experiments. The data were compared with the control group, treated with the same volume of deionized water (DI) as the stock of the test agent, and analyzed with one-way analysis of variance (ANOVA), with a significance level of 0.05.

3. Results

3.1. Effect of Pluronic L121, Tween 80, and L121-adjuvant on cell viability and the cell cycle

Apoptosis is a process of programmed death, with common features in many cell types. Cells undergoing apoptosis

show several morphological changes, including nuclear condensation, cell membrane blebbing, and DNA fragmentation [8]. To examine whether the surface-active agents in the L121-adjuvant had an adverse effect on cell viability, EL4 cells were treated for 24 h with various concentrations of Pluronic L121, Tween 80, or whole adjuvant, then examined for signs of cell death. In the case of Tween 80, three tests were used, namely the MTT and LDH release assays and PI staining; in the case of the other two agents, the MTT and LDH release assays could not be used due to the turbidity of the solutions formed at the experimental temperature of 37 °C, which is higher than the cloud point of Pluronic L121 (14 °C). Fig. 1 shows the results of cell cycle analysis by PI staining of EL4 cells after treatment with Pluronic L121 (3 μg/ml to 5 mg/ml). Flow cytometric analysis of ethanol fixed, PI-stained untreated control cells showed a typical diploid DNA peak (Fig. 1A). Treatment of the cells with concentrations of Pluronic L121 above 0.3 mg/ml resulted in the appearance of the hypodiploid DNA peak (Fig. 1F–I), a maximal increase in the sub-G1 phase of 50% being seen in those cells treated with 0.3 or 1 mg/ml of Pluronic L121 (Fig. 1F and G). A further increase in the Pluronic L121

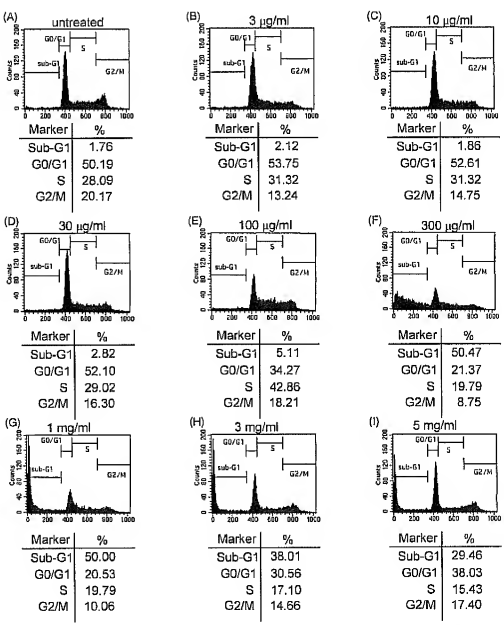

Fig. 1. Cell cycle analysis of EL4 cells after treatment with Pluronic L121. Approximately 1×10^6 cells were treated for 24 h with various concentrations (B–I) of Pluronic L121, fixed with 70% ethanol, and treated with 200 μl of phosphate buffer containing 0.2 M Na_2HPO_4, 0.1 M citric acid (pH 7.8) for 30 min. The cells were then centrifuged and stained with 1 ml of PI (80 μg/ml) staining buffer, followed by flow cytometric analysis. Untreated EL4 cells served as the control (A). The experiment was repeated three times with similar results.

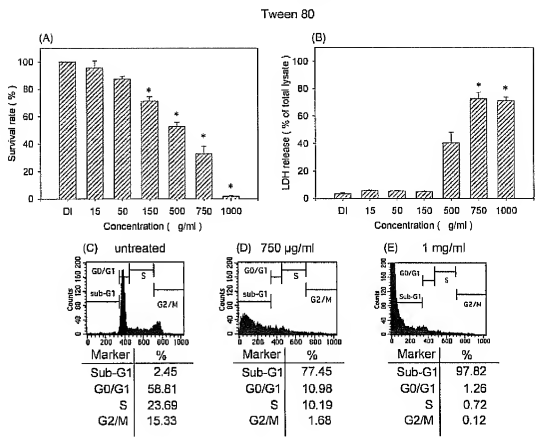

Fig. 2. Effect of Tween 80 on EL4 cell viability and cell cycles. (A) MTT assay. EL4 cells (1×10^5 cells/well) in 96-well plates were treated for 24 h with various concentrations of Tween 80, then MTT reagent was added for 1 h. The cells were then lysed with 1% Triton X-100 and the absorbance at 550 nm measured. (B) LDH release assay. Cells (5×10^3/well) in 96-well plates were treated for 24 h with various concentrations of Tween 80, then the supernatant was tested for LDH release as described in Section 2. The data are presented as the means ± S.E. for three separate experiments in triplicate. (*) Significantly different from the control group treated with the same volume of deionized water (DI) ($P < 0.05$, by one-way ANOVA). (C–E) PI staining. 1×10^6 cells were treated with various concentrations of Tween 80, then examined by PI staining and flow cytometry as described in Section 2. The experiment was repeated three times with similar results.

concentration to 5 mg/ml resulted in a decrease in the sub-G1 phase to 29.46% (Fig. 1I). These results indicated permeabilization of the cell membrane and DNA fragmentation after treatment with Pluronic L121, resulting in DNA leakage during the subsequent cell rinsing and staining. As a consequence, these cells showed a reduced DNA content in the G0/G1 phase and an increase in the percentage of cells in the sub-G1 phase as the Pluronic L121 concentration was increased above 100 μg/ml.

Similar results were obtained in EL4 cells after treatment for 24 h with Tween 80 at concentrations ranging from 15 μg/ml to 1 mg/ml. Fig. 2 shows the concentration-dependent decrease in viability of cells treated with Tween 80. The minimal cytotoxic concentration was determined to be approximately 150 μg/ml (Fig. 2A); at this concentration, cell viability was 28.6%. The amount of LDH release (Fig. 2B) in cells treated with Tween 80 at this concentration was negligible compared to control cells treated with 10% Triton X-100, reflecting the fact that these assays measure different aspects of cell viability. A further increase in the Tween 80 concentration up to 500 μg/ml resulted in approximately 40.4% LDH release. At 1 mg/ml of Tween 80, cell viability fell to 1.85%. Cell cycle analysis (Fig. 2C–E) showed the concomitant appearance of the sub-G1 phase at concentrations of Tween 80 of 750 μg/ml (77.5%) and 1 mg/ml (97.8%). Since the sub-G1 peak represents not only apoptotic cells, but also mechanically damaged cells and cells with lower DNA content, the results showed concentration-dependent DNA degradation following treatment with the surface-active agent.

To examine the cell cycle specificity of apoptosis in EL4 cells after treatment with L121-adjuvant, cells were incubated for 24 h in medium containing various doses of adjuvant, then examined by PI staining. Fig. 3 shows that treatment of EL4 cells with L121-adjuvant resulted in an increase in the G0/G1 and sub-G1 phases (Fig. 3A–C). However, higher concentrations resulted in cell cycle arrest in the G0/G1 phase (Fig. 3B–F), with a slight increase in cells in the sub-G1 phase, a concomitant decrease in those in S phase, and no significant changes in those in G2/M phase, indicating that cell proliferation was perturbed and that DNA replication and chromosomal events during

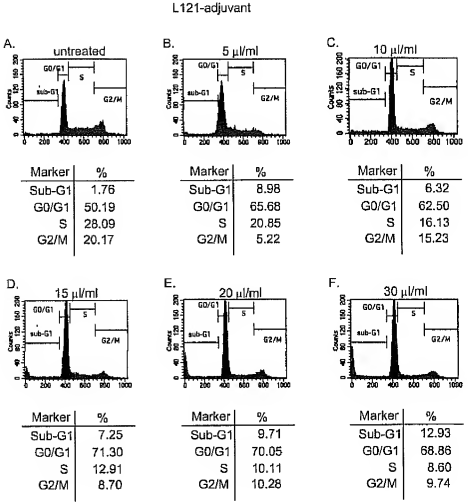

Fig. 3. Cell cycle analysis of EL4 cells after treatment with L121-adjuvant. Approximately 1×10^6 cells were treated with various doses of L121-adjuvant, then examined by PI staining and flow cytometry as described in Section 2. The experiment was repeated three times with similar results.

G0/G1 in EL4 cells were sensitive to treatment with L121-adjuvant.

3.2. Detection of apoptosis and necrosis with Annexin V/propidium iodide in EL4 cells after treatment with various test agents

Annexin V binds to PS, which is located predominantly on the internal leaflet of the plasma membrane of intact cells, but becomes exposed in apoptotic cells [8]. In addition, disruption of membrane integrity by surface-active agents makes the cell interior accessible to PI and double-staining with these reagents can be used to quantify both the early and late phases of apoptosis [27]. To quantify the extent of apoptosis and necrosis induced by surface-active agents, EL4 cells were therefore treated with the test agents, then double-stained with annexin V/PI, and analyzed by flow cytometry. As shown in Fig. 4, treatment of cells with increasing concentrations of Pluronic L121 (3 μg/ml to 5 mg/ml) resulted in a concentration-dependent increase in the percentage of apoptotic cells, whereas the percentage of necrotic cells reached a maximum at 1 mg/ml, then decreased. Similar results were obtained for Tween 80-treated cells (Fig. 5). Increasing the Tween 80 concentration from 15 μg/ml to 1 mg/ml resulted in an high percentage of apoptotic cells, with the maximum occurring at 1 mg/ml of Tween 80, with approximately 71.8% of the cells being apoptotic and 3.61% cells necrotic (Fig. 5H). The decrease in the percentage of necrotic cells at high concentrations of detergents (Figs. 4 and 5) can be attributed to the loss of a large number of necrotic cells during the rinsing and centrifugation steps after treatment with the surfactants at these concentrations. Treatment of EL4 cells with L121-adjuvant resulted in a concentration-dependent increase in the percentages of both apoptotic and necrotic cells (Fig. 6). PS is a key recognition molecule for phagocytosis by APCs, such as macrophages [14,28]. These results, showing that cell death and PS exposure were induced by the surface-active agents or adjuvant, strongly suggest that the apoptotic cells will be recognized by APCs and phagocytosed.

Comparison of roughly equivalent doses of the individual components and the whole adjuvant showed that most of the

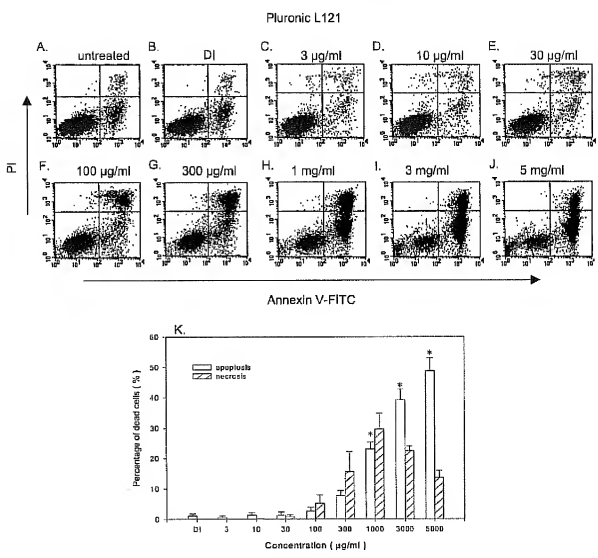

Fig. 4. Flow cytometric analysis of apoptosis and necrosis in EL4 cells treated with Pluronic L121. Approximately 1×10^6 EL4 cells were treated with various concentrations of Pluronic L121 for 24 h, then the cells were stained with annexin V and propidium iodide and analyzed by flow cytometry. The results shown in part (K) are the mean and S.E. for three independent experiments. (*) Significantly different from the control group treated with the same volume of deionized water (DI) ($P < 0.05$, by one-way ANOVA).

necrosis and apoptosis caused by the adjuvant could be attributed to Pluronic L121. The dose of 30 μl/ml of adjuvant, equivalent to 1.125 mg/ml of Pluronic L121 and 180 μg/ml of Tween 80, resulted in 18.2 and 30.0% apoptosis and necrosis, respectively, (Fig. 6), compared to the corresponding values of 23.0 and 29.7% for 1 mg/ml of Pluronic L121 (Fig. 4) and 3.97 and 1.56% for 150 μg/ml of Tween 80 (Fig. 5).

3.3. Examination of cell death by fluorescence microscopy

Apoptosis is characterized structurally by chromatin condensation, shrinkage of the cells, and DNA fragmentation. Disruption of membrane integrity by surfactants during apoptosis or necrosis makes the cell interior accessible to DNA-binding dyes. As shown in Fig. 7, surfactant-treated EL4 cells stained with Hoechst 33342 showed a different nuclear staining patterns to untreated control cells. Treatment of EL4 cells with Pluronic L121 or L121-adjuvant resulted in nuclear fragments (Fig. 7B-D), a characteristic features of apoptotic cell death, also seen in the positive control cells treated with staurosporine (Fig. 7E). Due to the strong cytolytic effect of Tween 80, only a small portion of the cells were recovered after Hoechst 33342 staining. These results are consistent with those obtained using flow cytometric analysis.

3.4. Effect of the general caspase inhibitor, zVAD-fmk

Caspase activation is an early step in cell death and precedes cell membrane disruption, while PS flipping is a consequence of caspase activation and a feature of apoptosis. To investigate the possible inhibitory effect of zVAD-fmk, a general caspase inhibitor, on PS exposure, EL4 cells were

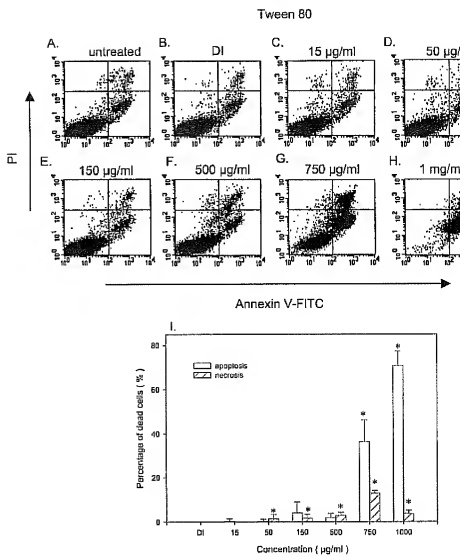

Fig. 5. Flow cytometric analysis of apoptosis and necrosis in EL4 cells treated with Tween 80. Approximately 1×10^6 EL4 cells were treated with various concentrations of Tween 80 for 24 h, then the cells were stained with annexin V and propidium iodide and analyzed by flow cytometry. The results shown in part (I) are the mean and S.E. for three independent experiments. (*) Significantly different from the control group treated with the same volume of deionized water (DI) ($P < 0.05$, by one-way ANOVA).

incubated with 50 μM zVAD-fmk for 2 h before treatment with the surfactants or L121-adjuvant, and were then stained with Annexin V/PI and analyzed by flow cytometry. Fig. 8 shows that pretreatment with 50 μM zVAD-fmk partially inhibited the apoptotic effect elicited by treatment with 1 mg/ml of Pluronic L121 (Fig. 8C and D), but not that elicited by either or 0.75 mg/ml of Tween 80 or 20 μl/ml of L121-adjuvant (Fig. 8E–H).

4. Discussion

Vaccine adjuvants are often emulsions containing at least two immiscible phases, oil and water. The surfactants in the emulsion serve as emulsifying agents adsorbing to the oil/water interface and generating a barrier against coalescence, thus stabilizing the dispersion. Although it has long been recognized that the immunological effects of vaccine adjuvants are related to the surface activity of the surfactants in the formulation [3], the relationship between the physicochemical properties of these surfactants and their immunological effect is unclear. Over the past two decades, the biological activities of adjuvants have been correlated with their surface activities through several physicochemical parameters, including the type of emulsion (oil-in-water or water-in-oil) formed or the HLB value, which measures the relative hydrophilic/hydrophobic properties of the surface-active agents. It is generally believed that surface-active components in vaccine adjuvants bind to the antigen and present it to APCs, thus stimulating the immune response. The immunological roles of surface-active agents

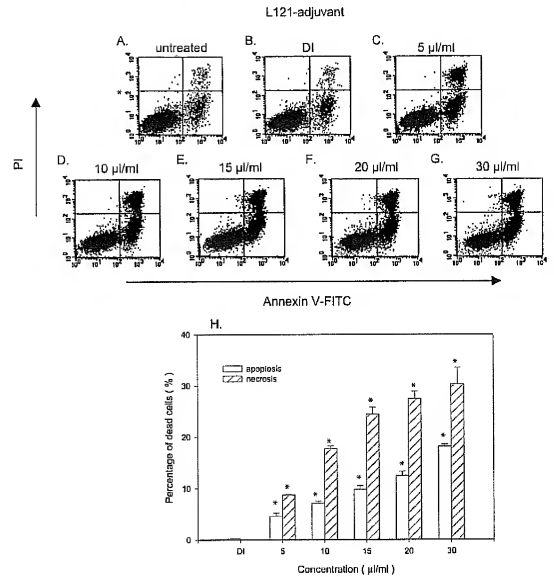

Fig. 6. Flow cytometric analysis of apoptosis and necrosis in EL4 cells treated with L121-adjuvant. Approximately 1×10^6 EL4 cells were treated with various doses of L121-adjuvant for 24 h, then the cells were stained with annexin V and propidium iodide and analyzed by flow cytometry. The results shown in part (H) are the mean and S.E. for three independent experiments. (*) Significantly different from the control group treated with the same volume of deionized water (DI) ($P < 0.05$, by one-way ANOVA).

in antigen presentation and processing however remain unknown.

Attempts have been made to use vaccine adjuvants to generate specific and effective priming of MHC class I-restricted CTLs, which play a key role in control of virus infection and tumors. Experimental evidence for antigen presentation by DCs have recently been obtained, showing that bone marrow-derived DCs can ingest antigens from apoptotic cells and cross-present antigens to class I-restricted $CD8^+$ CTLs [10,11]. Exposure to necrotic cells, on the other hand, provides signals for the maturation of immunostimulatory DCs and initiates immunity [12]. These findings raised the possibility that apoptosis or necrosis may be induced by emulsion-type vaccine adjuvants containing surfactants. Due to the amphiphilic nature of the surfactants, these molecules tend to adsorb not only to the oil/water interface in the emulsion, but also to biological membranes, resulting in an increase in surface pressure, leading to apoptosis and necrosis of the cells.

Apoptosis is a process of programmed cell death characterized by morphological as well as biochemical changes. In contrast, oncosis (primary necrosis), a passive degenerative process, was defined as the early stage of necrosis during which cells swell [8]. Necrosis, on the other hand, is signaled by irreversible changes in the nucleus after membrane disruption, thus include both oncosis and apoptotic necrosis (late apoptosis or secondary necrosis). The external phosphatidylserine (PS) exposure, an early event characteristic of apoptotic process, has been shown not to be specific to apoptotic cells, but also occurs in oncotic cells [29]. The cells

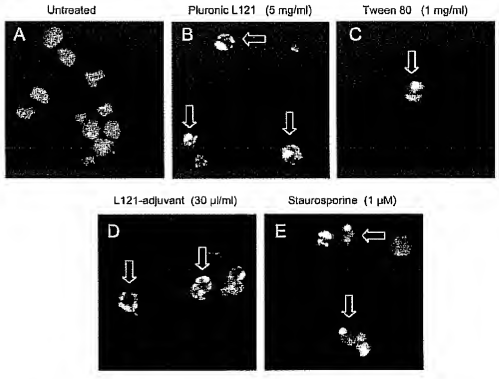

Fig. 7. Confocal photomicrographs of EL4 cells. (A) Untreated cells; (B–D) cells treated with 5 mg/ml of Pluronic L121 (B), 1 mg/ml of Tween 80 (C), or 30 μl/ml of L121-adjuvant (D). Staurosporine treated cells served as the positive controls for apoptosis (E). Cells were treated with the test agents for 24 h, stained with 0.1 μg/ml Hoechst 33342 for 15 min, centrifuged, washed with PBS, fixed with 4% paraformaldehyde for 15 min, then examined under a Zeiss fluorescence microscope. The experiment was repeated three times with similar results.

positively stained with Annexin V therefore include those undergoing early apoptosis, apoptotic necrosis, and oncotic necrosis. In the present study, supravital PI staining, in combination with Annexin V assay, was used to assess the loss of membrane integrity, including both early apoptosis (Ann V^+/PI^-) and necrosis (Ann V^+/PI^+).

The study utilized EL4 mainly because they are commonly used for studying apoptosis [30,31]. More importantly they are also a model target for studies on CTL induction by cross-presentation of antigen derived from apoptotic cells [11,12]. The surfactants used in the L121-adjuvant, Tween 80 and Pluronic L121, were shown to induce both apoptosis and necrosis in EL4 cells. Comparison of roughly equivalent doses of the individual components and the whole adjuvant showed that most of the necrosis and apoptosis caused by the adjuvant could be attributed to Pluronic L121 (Figs. 4–6). The results shown in Figs. 1–3 indicated that treatment of EL4 cells with the higher concentrations of the surfactants or adjuvant resulted in a marked increase in cell death, leading to the appearance of cells in the sub-G1 phase. The exposure of PS residues on the outside of the plasma membrane, allowing the binding of annexin V, which occurs prior to the loss of integrity of the plasma membrane, is often used to characterize early apoptosis. At concentrations of Tween 80 above 750 μg/ml, flow cytometric analysis revealed that the population of early apoptotic (Ann V^+/PI^-) cells greatly exceeded that of necrotic cells (Ann V^+/PI^+) (Fig. 5K) and similar results were seen with Pluronic L121-treated cells (Fig. 4K). In contrast, a higher percentage of necrosis (Fig. 6) was seen using high doses of L121-adjuvant. MTT reduction and LDH release assays showed that the surfactants and whole adjuvant induced cell membrane damage, leading to release of LDH into the culture medium. This necrotic effect was accompanied by apoptosis, characterized by the presence of Ann V^+/PI^- cells (Figs. 4–6) and DNA fragmentation (Fig. 7), suggesting PS externalization and showing that both types of cell death were induced by either surfactants alone or whole L121-adjuvant. It is noted that the extents of both apoptosis and necrosis, induced by Pluronic L121 (5 mg/ml) and L121-adjuvant (30 μl/ml), increased with time (data not shown), indicating the time-dependence of apoptosis and necrosis, which were in agreement with other studies [32].

Cell cycle analysis by flow cytometry indicated that L121-adjuvant arrested the cell cycle of EL4 cells in G0/G1 phase (Fig. 3). This arrest may be associated with inactivation of proteasomal proteins and thus altered cell cycle regulatory signaling. The concomitant decrease in the number of cells in S phase suggests that L121-adjuvant inhibits cell cycle progression from G0/G1 to S phase. The reason for the discrepancy between these results and those obtained for apoptosis using Annexin V/PI staining (Fig. 6) is unclear. Unlike Pluronic L121 and staurosporine induced apoptosis, pretreatment of EL4 cells with zVAD-fmk, a broad range

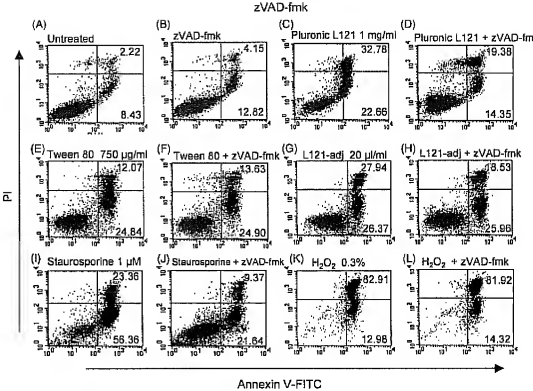

Fig. 8. Inhibitory effect of zVAD-fmk on apoptosis/necrosis induced by the surface-active agents in the vaccine adjuvant. EL4 cells at 1×10^6/ml/well in 12-well plates were treated with 50 mM zVAD-fmk for 2 h before incubation for 24 h with Pluronic L121, Tween 80, or L121-adjuvant. The cells were then subjected to annexin V/PI staining and flow cytometric analysis. The data presented are representative of three separate experiments. Staurosporine- or H_2O_2-treated cells served as the positive control for apoptotic or necrotic cells, respectively. The percentages of apoptotic or necrotic cells are shown, respectively, in the lower right and upper right quadrant.

caspase inhibitor, did not prevent the cell death induced by Tween 80 or L121-adjuvant (Fig. 8). These findings suggested that Tween 80 and L121-adjuvant induced apoptosis in EL4 cells via a "non-classical" caspase-independent pathway.

L121-adjuvant shares many similar features, including the compositions and surfactant concentrations, with the Syntex adjuvant formulation-1 (SAF-1) [17], which is composed of 5% (w/v) squalane, 2.5% Pluronic L121, 0.2% Tween 80, and various doses of Thr^1-MDP (muramyl dipeptide). Comparison of the in vitro results obtained in this study with the L121-adjuvant doses used in vivo however is difficult. Assuming no absorption occurs during the initial period after injection of regular doses of 30–100 μl and that adjuvant is distributed in a volume of less than 1 ml, the local dose of L121-adjuvant in the animals is expected to be higher than 30 μl/ml. Comparison with the results shown in Fig. 6, both apoptosis and necrosis are expected to take place in vivo at these doses of L121-adjuvant.

Although much research has been carried out on the immunostimulatory effect of adjuvants, cell death induced by adjuvants has been ignored as an important initiation signal for CTL induction. Most vaccine adjuvants reported to elicit CTL responses, including saponin-based immunostimulating complexes (ISCOM) [33–35], contain surface-active agents. The results shown in this study illustrate that the dose of vaccine adjuvant not only determines the extent, but also the type, of cell death and cell cycle arrest, which consequently modulates the pattern of the immune response. However, we cannot rule out the direct immunostimulatory effect and the delivery function of the adjuvants. The immunological effect of adjuvants is not only determined by the physical state of the active immunomodulating component, but also by the physicochemical properties of the carriers. The surfactants employed in the L121-adjuvant, particularly Pluronic L121, serve both as immunomodulating agents and carriers.

Apoptosis is important in several immunological responses. APCs, such as macrophages or DCs, are expected to phagocytose apoptotic cells and present apoptotic cell-derived antigens to T cells [11]. PS translocation in the plasma membrane is an important phagocytic signal for target APCs. Necrotic cells, on the other hand, serve as natural adjuvants to activate DCs by endogenous signals [36]. Although recent studies demonstrated that DCs also acquire antigens from live cells and cross-present antigens to MHC class I-restricted CTL [37], the presence of dying cells was shown to facilitate uptake of the antigens by the DCs and subsequently implement cross-presentation of antigens to both $CD4^+$ and $CD8^+$ T cells [38]. In this

context, surfactant-induced apoptosis and necrosis have several implications for the immunogenicity and immune responses elicited by vaccine adjuvants containing them. These results potentially shed light on two important issues in the design of vaccine delivery systems: (1) how antigens are bound, processed, and acquired by APCs, particularly DCs, via adjuvant-induced apoptotic and necrotic cells; and (2) how these dead cells induce the differentiation and maturation of DCs and activate CTLs. Overall, the results presented in this study demonstrate that exposure to surfactant-containing vaccine adjuvants induces cell apoptosis and necrosis in a concentration-dependent manner. These findings, along with the established notion of antigen acquisition and cross-presentation by APCs, suggest rationales for cross-therapeutic immunization and better manipulation of vaccine adjuvants for the delivery of exogenous antigens to $CD8^+$, class I-restricted CTLs.

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References

[1] Glenny AT, Buttle GAH, Stevens MF. Rate of disappearance of diphtheria toxoid injected into rabbits and guinea pigs: toxoid precipitated with alum. J Pathol Bacteriol 1931;34:267–75.
[2] Freund J. Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil. Proc Soc Exp Biol Med 1937;37: 509–13.
[3] Hunter R, Strickland F, Kezdy F. The adjuvant activity of nonionic block polymer surfactants. I. The role of hydrophile-lipophile balance. J Immunol 1981;127(3):1244–50.
[4] Rueyhaudhuri S, Tonks M, Carbone F, Ryskamp T, Morrow WJ, Hanna N. Induction of antigen-specific class I-restricted cytotoxic T cells by soluble proteins in vivo. Proc Natl Acad Sci USA 1992;89(17):8308–12.
[5] Sheikh NA, Rajanandhanan P, Attard GS, Morrow WJ. Generation of antigen specific $CD8^+$ cytotoxic T cells following immunization with soluble protein formulated with novel glycoside adjuvants. Vaccine 1999;17(23–24):2974–82.
[6] Strupp W, Weidinger G, Scheller C, Ehret R, Ohnimus H, Girsehick H, et al. Treatment of cells with detergent activates caspases and induces apoptotic cell death. J Membr Biol 2000;175(3):181–9.
[7] Cohen JJ, Duke RC, Fadok VA, Sellins KS. Apoptosis and programmed cell death in immunity. Annu Rev Immunol 1992;10: 267–93.
[8] Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol 1995;146(1):3–15.
[9] den Haan JM, Lehar SM, Bevan MJ. $CD8^+$ but not $CD8^-$ dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med 2000;192(12):1685–96.
[10] Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, Silverstein RL, et al. Immature dendritic cells phagocytose apoptotic cells via $\alpha_v\beta_5$ and CD36, and cross-present antigens to cytotoxic T lymphocytes. J Exp Med 1998;188(7):1359–68.
[11] Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature 1998;392(6671):86–9.
[12] Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med 2000;191(3):423–34.
[13] Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 1992;148(7):2207–16.
[14] Verboven E, Schlegel RA, Williamson P. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. J Exp Med 1995;182(5):1597–601.
[15] Rajanandhanan P, Attard GS, Sheikh NA, Morrow WJ. Novel aggregate structure adjuvants modulate lymphocyte proliferation and Th1 and Th2 cytokine profiles in ovalbumin immunized mice. Vaccine 1999;18(1–2):140–52.
[16] Hariharan K, Hanna N. Development and application of PROVAX (TM) adjuvant formulation for subunit cancer vaccines. Adv Drug Deliv Rev 1998;32(3):187–97.
[17] Allison AC, Byars NE. An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and of cell-mediated immunity. J Immunol Methods 1986;95(2):157–68.
[18] Hunter RL, Bennett B. The adjuvant activity of nonionic block polymer surfactants. II. Antibody formation and inflammation related to the structure of triblock and oetablock copolymers. J Immunol 1984;133(6):3167–75.
[19] Ott G, Barchfeld GL, Van Nest G. Enhancement of humoral response against human influenza vaccine with the simple submicron oil/water emulsion adjuvant MF59. Vaccine 1995;13(16):1557–62.
[20] Cataldo DM, VanNest G. The adjuvant MF59 increases the immunogenicity and protective efficacy of subunit influenza vaccine in mice. Vaccine 1997;15(16):1710–5.
[21] Shevach EM, Stobo JD, Green I. Immunoglobulin and theta-bearing murine leukemias and lymphomas. J Immunol 1972;108(5):1146–51.
[22] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65(1–2):55–63.
[23] Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods 1988;115(1):61–9.
[24] Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. J Immunol Methods 1983;64(3):313–20.
[25] Corr M, von Damm A, Lee DJ, Tighe H. In vivo priming by DNA injection occurs predominantly by antigen transfer. J Immunol 1999;163(9):4721–7.
[26] Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139(2):271–9.
[27] Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 1994;84(5):1415–20.
[28] Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death Differ 1998;5(7):551–62.
[29] Lecoeur H, Prévost M-C, Gougeon M-L. Oncosis is associated with exposure of phosphatidylserine residues on the outside layer of the plasma membrane: a reconsideration of the specificity of the annexin V/propidium iodide assay. Cytometry 2001;44:65–72.

[30] Jessel R, Haeriel S, Soceiu C, Tykhonova S, Diehl HA. Kinetics of apoptotic markers in exogeneously induced apoptosis of EL4 cells. J Cell Mol Med 2002;6(1):82–92.
[31] Telford WG, Komoriya A, Packard BZ. Detection of localized caspase activity in early apoptotic cells by laser scanning cytometry. Cytometry 2002;47(2):81–8.
[32] Sun XM, Snowden RT, Skilleter DN, Dinsdale D, Ormerod MG, Cohen GM. A flow-cytometric method for the separation and quantitation of normal and apoptotic thymocytes. Anal Biochem 1992;204:351–6.
[33] Morein B, Sundquist B, Hoglund S, Dalsgaard K, Osterhaus A. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature 1984;308(5958): 457–60.
[34] Morein B. Iscoms. Vet Microbiol 1990;23(1–4):79–84.
[35] Morein B. The iscom: an immunostimulating system. Immunol Lett 1990;25(1–3):281–3.
[36] Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. Nat Med 1999;5(11):1249–55.
[37] Harshyne LA, Watkins SC, Gambotto A, Barratt-Boyes SM. Dendritic cells acquire antigens from live cells for cross-presentation to CTL. J Immunol 2001;166(6):3717–23.
[38] Iyoda T, Shimoyama S, Liu K, Omatsu Y, Akiyama Y, Maeda Y, et al. J Exp Med 2002;195(10):1289–302.